

Study of the thymic-derived or Independant nature of the mouse spleen cells induced to proliferate in culture by various mitogens and antigens

(PROLIFERATION IN VITRO DES LYMPHOCYTES
T (THYMO DEPENDANTS) ET B (THYMO INDEPENDANTS)
APRES STIMULATION PAR DIVERS ANTIGENES ET MITOGENES)

THESE

présentée à la Faculté de médecine de l'Université de Genève
pour obtenir le grade de docteur en médecine

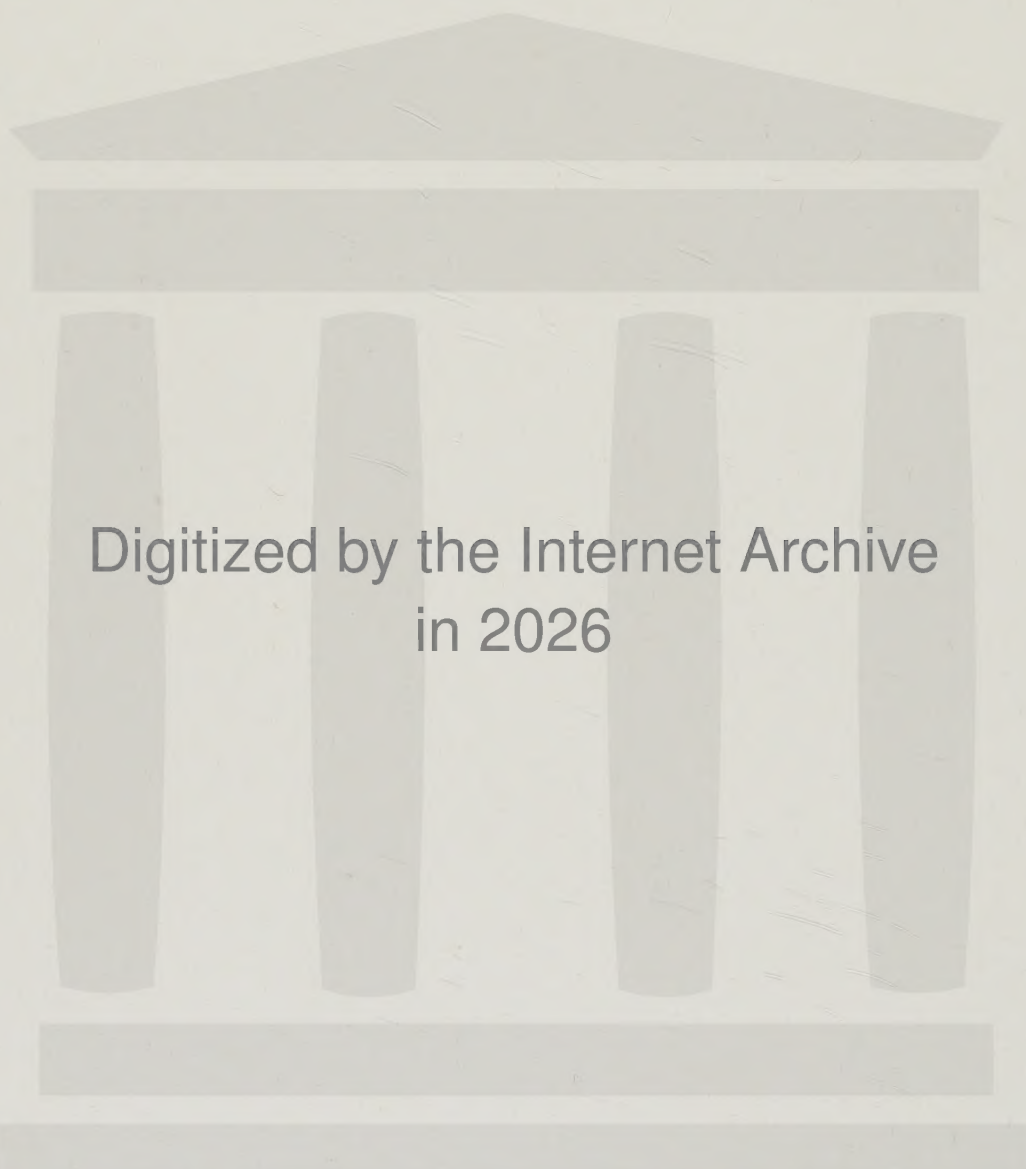
par

Pierre-François PIGUET

de

Genève

Thèse No 3360



Digitized by the Internet Archive
in 2026

https://archive.org/details/IA41545902_0044

Study of the thymic-derived or Independant nature of the mouse spleen cells induced to proliferate in culture by various mitogens and antigens

(PROLIFERATION IN VITRO DES LYMPHOCYTES
T (THYMO DEPENDANTS) ET B (THYMO INDEPENDANTS)
APRES STIMULATION PAR DIVERS ANTIGENES ET MITOGENES)

T H E S E

présentée à la Faculté de médecine de l'Université de Genève
pour obtenir le grade de docteur en médecine

par

Pierre-François PIGUET

de

Genève

Thèse No 3360

La Faculté de médecine, sur le préavis du professeur Pierre Vassalli, autorise l'impression de la présente thèse, sans prétendre par là émettre d'opinion sur les propositions qui y sont énoncées.

Genève, le 19 juillet 1973

Le doyen :
William Geisendorf

Thèse No 3360



(European Journal Immunology, vol. 3, 1973, No. 8).

J'exprime ici ma gratitude au professeur P. Vassalli qui a rendu possible ce travail.

Study of the thymic-derived or -independent nature of mouse spleen cells induced to proliferate in culture by various mitogens and antigens

The nature of mouse lymphoid cells induced to proliferate *in vitro* by a number of stimulating agents has been explored by 1) karyotypic analysis of the mitoses observed in stimulated spleen cell cultures (arrested on sequential days) of chimaeras bearing chromosomally marked T cells; 2) comparison between the response in DNA synthesis of total spleen cells and of spleen cells depleted of thymus-dependent lymphocytes by treatment with anti-MSLA (anti-mouse-specific lymphocyte antigen) + C, and 3) study of the response of cortisone-resistant thymocytes (CRT) to some of these agents. Pokeweed mitogen stimulated both B and T cells equally and simultaneously. Rabbit anti-mouse Ig induced moderate proliferation of B cells exclusively. The response to lipopolysaccharide was entirely B cell-dependent, but evidence was found of some T cell proliferation following or accompanying the stimulation of B cells. A similar observation was made in the case of "primary" or "secondary" *in vitro* response to bacteriophage T4. Good stimulation with sheep red blood cell stromas or mitogen-treated xenogenic spleen cells were observed only in cultures from primed animals, and consisted of a mixed B and T cell proliferation with a majority of B mitoses, even at the earlier stages of the response. That this "secondary" response was not totally T cell-dependent was suggested by the observation of a stimulatory effect of these antigens on T-depleted spleen cell cultures which were found in parallel experiments to be totally unresponsive to PHA. Finally, using chimeric mice treated *in vivo* with vinblastine prior to establishment of the spleen cell cultures, evidence was obtained that part of the B cells stimulated to divide *in vitro* by several of these mitogens belong to a rapidly dividing cell population *in vivo*.

1. Introduction

The thymic-dependent (T) or -independent (B) nature of lymphoid cells stimulated to divide *in vitro* under the influence of various mitogens or specific antigens can be studied by using spleen cultures of mouse radiation chimaeras bearing chromosomally marked T cells [1, 2]. When spleen cell cultures of such radiation chimaeras were stimulated by phytohemagglutinin (PHA) or by allogenic spleen cells (mixed lymphocyte culture, MLC), karyotypic analyses showed a marked predominance of T mitoses on days 2 and 3 of the cultures, followed by a strong predominance of B mitoses on days 4 and 5 [2]. Spleen cells depleted of T cells were not stimulated by PHA or in MLC; a B cell mitotic response to PHA could be restored by a very small number of T cells or by the cell-free supernatant of a 24 h MLC [2]. These results are in agreement with the concept that under certain conditions, B cell stimulation requires the presence of a soluble factor released by stimulated T cells [3–5]. The present work concerns the nature of the lymphoid cell proliferation induced *in vitro* by a number of other stimulating agents. The effects of pokeweed mitogen (PWM), *E. Coli* lipopolysaccharide (LPS), rabbit anti-mouse immunoglobulin (RaMig) antiserum, bacteriophage T4, sheep red blood cell (SRBC) stroma and xenogenic lymphoid cells have been explored by:

- 1) karyotypic analysis of the mitoses observed in stimulated spleen cell cultures of chimaeras bearing chromosomally marked T cells;
- 2) comparison between the response of total spleen cells and of T-depleted spleen cells and, when feasible,
- 3) capacity of immunocompetent thymic cells (cortisone-resistant thymocytes (CRT)) to be stimulated by some of these agents.

2. Materials and methods

2.1. Preparation of chimaeras bearing chromosomally marked T cells

CBA/ca or CBA/H-T6T6 (Jackson Laboratory, Bar Harbor, Maine) were thymectomized at 4–8 weeks of age, subjected 2–4 weeks later to 850–950 rads of total body irradiation, and injected within 24 h with 5×10^6 bone marrow cells treated with mouse AKR anti- Θ C₃H serum and rabbit complement (C). Within 4–8 days, $4-8 \times 10^7$ thymocytes (from 4–6 week old donors) were injected intravenously. Three types of CBA/ca-CBA/H-T6T6 chimaeras were prepared by reconstitution after irradiation: (a) with bone marrow of the host karyotype and thymocytes of the other karyotype (T/H + BM mice, *i.e.* mice in which injected thymocytes have one karyotype, while bone marrow and residual host cells have the other); (b) with bone marrow of the other karyotype and thymocytes of the host karyotype (T + H/BM mice); (c) with bone marrow of (CBA/ca $\times$ CBA/H-T6T6)_{F1} hybrids (bearing a single T6 chromosome) and thymocytes of the other karyotype (T/H/BM mice, *i.e.* mice in which three karyotypes can be found, thymocytes bearing two T6 chromosomes and residual host cells none (or vice versa), and bone marrow cells only one T6). Chimaeras were utilized 4–8 weeks after reconstitution.

Correspondence: Pierre-François Piguet, Department of Pathology, University of Geneva, 40 Bd. de la Cluse, CH-1211 Geneva 4, Switzerland

Abbreviations: B cell: Bone-marrow derived lymphocyte T cell: Thymus-derived lymphocyte MLC: Mixed lymphocyte culture PHA: Phytohemagglutinin PWM: Pokeweed mitogen SRBC: Sheep red blood cell(s) LPS: Lipopolysaccharide T4: Bacteriophage T4 anti-MSLA: Rabbit anti-mouse-specific lymphocyte antigen NRS: Normal rabbit serum RaMig: Rabbit anti-mouse immunoglobulin FCS: Fetal calf serum CRT: Cortisone-resistant thymocytes SI: Stimulation index

2.2. Preparation of spleen cells depleted in T lymphocytes

10^7 nucleated spleen cells (10^7 /ml) in Hanks' balanced salt solution (BSS) were incubated with rabbit anti-MSLA (mouse-specific lymphocyte antigen) [6] at a concentration of 1 : 10 for 30 min at 37 °C followed by a second 30 min incubation containing 1 : 12 agarose-absorbed rabbit serum as a source of complement [6]. After two further washings in sterile Hanks' BSS, the nucleated cell suspension contained 70–80 % of viable cells, as judged by trypan blue dye exclusion test, and were used for culture.

2.3. Cortisone-resistant thymocytes

These were prepared according to Blomgren and Andersson [7].

2.4. Cultures

Viable nucleated spleen cells ($1 \times 10^6 - 2 \times 10^6$) or CRT were distributed to each culture tube (Falcon plastics, Los Angeles, Calif., 12 x 75 mm) in 1 ml of medium RPMI 1640 (M.B.A. Microbiological Associates, Bethesda, Maryland) usually containing 6 % fetal calf serum (FCS, Flow Laboratories Ltd, Irvine, Scotland) or 6 % fresh Wistar rat serum. Cultures were kept for two to five days at 37 °C in a humidified atmosphere (95 % air, 5 % CO₂). Parallel cultures were used for (a) karyotypic analyses [8] with usually around 50 mitoses analyzed per culture, with a minimum of 30 under less favorable conditions (b) DNA synthesis: 1 μ Ci of [³H]thymidine (5 Ci/mmole, Radiochemical Centre, Amersham, England) was added to cultures a few hours before harvesting; the cells were collected by centrifugation, precipitated with 0.5 M perchloric acid, the precipitates collected by membrane filtrations and further washed with perchloric acid and ethanol; the filters were incubated in 0.3 ml NCS (Amersham-Searle Corporation, Des Plaines, Illinois) for 2 h at 40 °C, toluene-Omnifluor was added and radioactivity determined in a Beckman L-250 liquid scintillation counter. The results were expressed as mean of cpm per tube of duplicate cultures.

2.5. Stimulating agents

(a) PWM (Pokeweed mitogen, Gibco, Grand Island, New York) reconstituted as prescribed, was used at the optimal dose of 40 μ l/ml. (b) LPS (*E. Coli* lipopolysaccharide 0127:B8, Difco Laboratories, Detroit, Michigan) was detoxified before use by heating for 60 min at 90 °C, and used at an optimal concentration of 10–60 μ g/ml. (c) RaMIg (rabbit anti-mouse Ig) was prepared as described elsewhere [6], absorbed on mouse thymocytes and used at a final concentration of 8 % plus 2 % FCS. (d) SRBC stromas were prepared by hypotonic lysis of SRBC and repeated centrifugations (15 min at 30 00 x g) until the supernatant was free of hemoglobin. The pellet was resuspended in Hanks' BSS sterilized by exposure to ultraviolet light, and the optimal stimulatory dose determined. (e) Phages T4 were purified on sucrose gradient according to Dickson et al. [9] and used at a final concentration of 15 μ g protein/ml. Xenogenic lymph node cells were obtained from Wistar rats or random-bred guinea pigs: 10×10^6 cells/ml were pretreated for 30 min at 37 °C in Hanks' BSS containing 50 μ g mitomycin-C (Sigma Chemical Co., St. Louis, Mo.), washed twice, and added to mouse spleen cultures at a final concentration of 4×10^5 cells/ml. The stimulation indexes (S.I.) were calculated as the ratios of radioactivity incorporated in stimulated over "background" cultures.

3. Results

3.1. Problems concerning the types of chimaera used

Chimaeras of the T/H/BM type, where residual host cells and injected thymus as well as bone marrow cells can be distinguished by the existence of three different karyotypes (either no, or one, or two T6 chromosomes) showed that a few mitoses were of host origin. These host mitoses were considered as T mitoses [10], although they were observed not only on the first days of culture with PHA, but also with agents which stimulate mainly B cells, such as LPS and phage T4 (see below). It appears certain that residual host T cells do exist, however, since 10 chimaeras which were reconstituted only with anti- Θ -treated bone marrow cells, and not with thymocytes, showed a definite, although decreased, *in vitro* response to PHA (SI varying between 1.5 to 10). The host cells represented, on the average, 58 % of the dividing cells on the second day of culture, and 39 % on the third. Since destruction of T cells *in vitro* by anti-MSLA + C leads to total lack of responsiveness to PHA (Table 2), these experiments strongly suggest that thymectomized, x-irradiated mice still contain residual host T cells capable of responding to some stimuli. Consequently, if most host lymphoid cells surviving radiation are indeed T cells, when chimaeras with 2 markers only were used, the T + H/BM chimaeras were considered to reflect more accurately the B or T nature of the dividing cells than the T/H + BM chimaeras. Whether the interpretation of the host mitoses was always correct or not certainly did not affect the final interpretation of the results, since this would not significantly alter the average percentages of T mitoses observed under the influence of a given mitogen, as presented in Table 4. It may be added that spleen cell cultures of almost all chimaeras were performed not only in the presence of the stimulating agent being tested, but also, in parallel cultures, in the presence of PHA, in order to ascertain that the karyotypic results on day 2 or 3 after PHA corresponded to the average values already reported [2], showing a strong majority of T cells. The average percentage of T mitoses was close to 80 % on the third day [2], except for a few mice which, shortly after immunization with SRBC, showed a decreased response to PHA (an observation also made by Adler [11]) and a percentage of T mitoses unusually low. This control made clear that the various types of mitotic responses reported in Table 1 really reflected the action of the mitogen and not some peculiarity of the chimaeras themselves (for instance, poor reconstitution in karyotypically labeled T cells).

3.2. Problems related to the culture medium used

Cultures were performed either in fetal calf or in rat serum. Unstimulated "background" cultures in FCS usually showed a much higher level of DNA synthesis than similar cultures in rat serum, and the total DNA synthesis in stimulated cultures was also higher in FCS than in rat serum. However, due to the low "background" level, the SI was usually higher in rat serum. The differences between rat and fetal calf serum seem to result from an enhancing activity of the latter on the cultures and not from a lesser ability of the former to support the cultures and/or the proliferation of a given type of lymphoid cell, as shown by various controls performed with cultures lasting five days. The use of either serum was not accompanied by any striking differences in the karyotype of dividing cells in cultures stimulated by the same agent. The nonstimulated cultures in FCS showed an average of 27 % T mitoses on day 3 [2], while in rat serum, mitoses were too sparse for an accurate karyotypic analysis.

Table 1. Karyotypic analysis of spleen cell cultures stimulated by various mitogens

Stimulant	Type of chimaera and of culture	Day 2		Day 3		Day 4	
		% T mitoses ^{a)}	S.I.	% T mitoses	S.I.	% T mitoses	S.I.
PWM	1) T+H/BM, FCS (average results)	45 [4] (32-63)	20-30	58 [3] (48-76)	10-20	65 [3] (52-72)	7
RaMIg	2) T+H/BM, FCS (indiv. exp.)	45	29	58	20	52	7
	T/BM+H and T+H/BM in 6-8 % RaMIg + 2 % FCS ^{b)} (average results)		N.D. ^{f)}	12 [7] (6-16)	2-6		N.D.
LPS	T/H/BM and T+H/BM, FCS (average results)	9.5 [2] (3-16)	20	16 [4] (14-19)	10		N.D.
Phage T4, unprimed mice	T/H/BM, T+H/BM and T/H+BM, FCS (average results)		N.D.	17 [8] (4-28)	4-5		N.D.
Phage T4, primed mice ^{c)}	T/H/BM and T+H/BM, FCS (average results)	26 [2] (20-32)	N.D.	26 [8] (12-53)	5-20	22 [1]	N.D.
SRBC-primed mice ^{d)}	1) T+H/BM, FCS (average results)	22.5 [2] (20-25)	N.D.	27 [5] (22-46)	2-3	20.5 [2] (20-21)	N.D.
	2) T+H/BM, FCS (indiv. exp. 14 days after priming)	20	N.D.	26	3	20	N.D.
	3) T+H/BM, rat serum (average results)	25 [7] (6-45)	5-20	38 [7] (31-44)	5-26	27 [7] (7-45)	5-20
	4) T+H/BM, rat serum (indiv. exp. 13 days after priming)	20	19	43	21	12	20
Xenogenic LN cells (mitomycin-treated), primed mice ^{e)}	1) T+H/BM, rat serum (average results)	24 [2] (22-26)	2-4	25 [6] (16-38)	2-6	37 [3] (25-39)	3
	2) T+H/BM, rat serum (indiv. exp. with rat cells)	21	4	30	4	28	3

a) Numbers in parantheses indicate the range of values observed; numbers in brackets indicate number of experiments.

b) "Background cultures" used to determine S.I. were, in these experiments, performed in normal rabbit serum (absorbed on mouse thymocytes) + 2 % FCS.

c) 40-170 μ g phage T4 i.p. 7-21 days before sacrifice.

d) $10^6 - 10^7$ SRBC i.v. 8-21 days before sacrifice.

e) 10^7 rat or guinea pig spleen cells, 7-13 days before sacrifice; LN = Lymph node.

f) N.D. = not done.

3.3. Pokeweed mitogen (PWM)

PWM gave a strong and early (day 2) stimulation, and the karyotypic analysis on sequential days showed that both B and T cells were stimulated about equally and simultaneously (Table 1). The capacity of PWM to stimulate both B and

T cells is apparent from the experiments reported in Table 2, where destruction of spleen T cells by treatment with anti-MSLA + C only decreased the response to PWM, while completely suppressing the PHA stimulation, and in Table 3 where CRT responded strongly to PWM.

Table 2. Results of [³H]thymidine incorporation in 3 day cultures of total spleen cells treated with anti-MSLA (aMSLA) + C in the presence of various stimulating agents

Stimulating agent and culture medium	Treatment of spleen cells before culture	Unstimulated cultures	Stimulated cultures	PHA in parallel cultures (control)		
		(cpm)	(cpm)	S.I.	(cpm)	S.I.
1) PWM in FCS	0	10000	110000	11	340000	34
	aMSLA + C	16000	55000	3.5	7000	< 1
2) LPS in FCS	aMSLA + C	6400	93000	13	4200	< 1
3) T4 in FCS (unprimed mice)	0	10000	67000	6.7	340000	34
	aMSLA + C	16000	104000	7	7000	< 1
4) SRBC (primed mice) a) in FCS	0	16000	135000	9	172000	11
	aMSLA + C	16000	167000	10	14500	< 1
	0	9500	94000	9	220000	23
	aMSLA + C	12000	74000	6	3000	< 1
c) in rat serum	0	800	10000	12	27000	34
	aMSLA + C	350	2300	6.5	800	2
d) in rat serum	0	700	21500	30	35000	50
	aMSLA + C	600	2500	4	500	< 1
5) Xenogenic (rat) spleen cells (primed mice) in FCS	0	14000	67000	4.8	124000	9
	aMSLA + C	14000	28000	2	14000	1

3.4. Rabbit anti-mouse Ig serum (RaMIg)

RaMIg serum induced a moderate stimulation of DNA synthesis which, as judged on the third day of culture (Table 1), consisted mainly of B cells. The culture with the highest SI [6] had only 7 % T mitoses. In several experiments it was found that RaMIg did not induce any stimulation of CRT (Table 3).

Table 3. [³H]thymidine incorporation in cultures of CR thymocytes in the presence of various mitogens. Cultures performed in rat serum with CRT of CBA/J mice and arrested on day 3.

Mitogen added	[³ H]thymidine incorporation	
	(cpm)	(cpm)
Background	150	300
LPS	200	—
PHA	34000	24000
RaMIg (2 %)	200	—
PWM	—	47000
T4	—	200

3.5. LPS

Cultures with LPS showed a stronger stimulation of DNA synthesis on day 2 than on day 3, and a marked predominance of B mitoses. However, in view of the strong stimulation, an average percentage of 16 % T mitoses on day 3 does not exclude the possibility that some T cells were also stimulated (see below). The lack of T cell requirement for B cell stimulation by LPS was evident in a number of experiments where, after destruction of T cells, cells of normal spleen responded very strongly to LPS (Table 2). On the other hand, CRT did not respond at all to LPS (Table 3).

3.6. Bacteriophage T4

Cultures with phage T4 showed good stimulation of DNA synthesis, which was always stronger in primed than in virgin mice. A strong predominance of B mitoses was observed in either situation; however, there were more T mitoses in primed mice. Although it is difficult to appraise the significance of this moderate difference, it appeared to be real. In five different pairs of matched chimaeras, (chimaeras prepared at the same time and in the same conditions) with one immunized with T4 and one not immunized, only in one instance was the percentage of T mitoses on the third day of culture similar in the same pair, while in the other four cases the cultures of the unprimed chimearas had less T mitoses than those of the primed mice (7 vs. 21, 10 vs. 20, 4 vs. 14 and 19 vs. 50). This suggests that the priming stimulated some T cells which are subsequently triggered into division by T4 in the culture. The T cells, however, are not necessary for the response of spleen cells to T4 (Table 2) and T4 is unable to stimulate CRT (Table 3). It should be pointed out that the stimulation of the cultures by T4 cannot result from a contamination of the T4 by bacterial membranes containing LPS, since the purified T4 suspensions were devoid of contaminating membranes, as judged by electron microscopic observation of these suspensions by negative staining [12].

3.7. SRBC

SRBC stroma in the cultures led to an inconstant, weak or absent stimulation in unprimed mice, as reported by Adler

et al. [13]. With mice primed 8 to 21 days before cultures, definite and constant stimulation was observed. Results of cultures in FCS and rat serum are presented separately, because it has been reported that some FCS contain a factor which restores the *in vitro* immune response to SRBC of the spleen of neonatally thymectomized mice [14]. With FCS (Table 2), the cultures showed a predominance of B mitoses from the second to the 4th day, but there can be little doubt that T cells were stimulated to divide, since in one instance a percentage of T mitoses close to 50 % was observed on the 3rd day. With rat serum, where the 7 chimaeras studied had cultures performed on sequential days, the SI were much stronger, the strongest being 26 times the background on the 3rd day of culture. Here, too, the mitoses showed a stimulation of both B and T cells, the highest observed percentage of T cell mitoses being 45 %. It was also clear from results of cultures performed on sequential days, in rat serum as well as in FCS, that there was no burst of T mitoses preceeding B mitoses, in contrast to what was observed with PHA and MLC [2].

When experiments were performed to compare the effect of T cell depletion on the response to SRBC stroma of cultures of primed mice, the results definitely indicated that B cell stimulation could be observed in the absence of T cells. In cultures performed in FCS, the stimulation of DNA synthesis of T-depleted cells by SRBC stroma was about as strong as that of total spleen cells of the same mice. Two such experiments are shown in Table 2. In a total of 4 experiments (with SI ranging from 2.5 to 9, average 6), the SI of T-depleted cells varied between 70 to 100 % of that of total spleen cells. In rat serum, T-depleted cells responded somewhat less to the antigen than total spleen cells: two experiments are shown in Table 2. Of a total of 4 experiments (with SI ranging from 4 to 30, average 12), the SI of T-depleted cells varied between 13 and 80 % of that of total spleen cells.

3.8. Xenogenic lymphoid cells

Cultures of unprimed chimaeras with mitomycin-treated xenogenic lymphoid cells (rat or guinea pig spleen cells) gave stimulation too low for proper analysis of mitoses, but cultures of primed chimaeras responded well under the same conditions (Table 1). From day 2 to 4, the mitoses were predominantly B, but the percentage of T mitoses was such, reaching almost 40 % in some cultures, that a stimulation of T cells appears certain. T cells of the spleen of primed mice were not found to be necessary, for stimulation by xenogenic cells, although the response of T-depleted cells was always smaller than that of total spleen cells. One experiment is shown in Table 2 and the entire results can be summarized as follows: in 3 cultures performed in FCS and stimulated by mitomycin-treated rat cells, the SI of total spleen cells were 3.2, 3.2 and 4.8, while the SI of spleen cells previously treated with anti-MSLA + C were, respectively, 85, 56 and 45 % of these values. In 3 cultures performed in rat serum and stimulated by mitomycin-treated guinea pig cells, with SI of 3,5 and 5, the T-depleted spleen cells showed SI in 2 cultures which were 53 and 40 % of those of total spleen cells. There was no stimulation in the last culture.

3.9. Spleen cell cultures established from mice treated *in vivo* with vinblastine

In order to determine if the cells stimulated to divide *in vitro* belonged preferentially to a class of lymphocytes dividing rapidly *in vivo*, vinblastine was injected (Velban, Eli Lilly and Co., Indianapolis, Ind., 0.06 mg i.p. 3 to 4 times at 8 h

intervals before sacrifice), since such treatment kills some of the rapidly dividing cells *in vivo* by arresting them in metaphase [15]. To appraise the effect of vinblastine, 4 mice were injected twice with 1 μ Ci/ml of [3 H]thymidine at 2 h intervals to label some of the rapidly dividing cells, and 2 of them were injected 1 h after the last injection with vinblastine as outlined above. In the vinblastine-treated mice, the [3 H]thymidine/ 10^6 cells incorporated into DNA at the time of sacrifice was about 20 % of that obtained from nontreated mice. This result indicates that the treatment was effective in killing some of the "rapidly dividing" cells which had been labeled by the injection of [3 H]thymidine. Compared to that of untreated mice, the total DNA synthesis in cultures from vinblastine-treated mice was 2 to 4 times lower in unstimulated cultures, usually unchanged in cultures with PHA, and decreased (about 50 %) with LPS and phage T4. However, a striking difference was observed when the karyotype of the dividing cells were studied: the percentage of T mitoses was strongly increased in cultures stimulated by LPS, T4 and SRBC stromas, the only stimulating agents studied in these conditions (Table 4) in addition to PHA. Although there were only two experiments per stimulating agent, the increase in T mitoses on day 3 appears unequivocal, since the percentages of T mitoses in the cultures from the vinblastine-treated animals were much higher than the highest percentages ever found in similar conditions in the series of cultures performed with untreated animals (see Table 1 for comparison). Two PHA-stimulated cultures of vinblastine-treated animals were also studied on day 4: both of them showed 80 % of T mitoses, in contrast to an average value of 26 % T mitoses in cultures of untreated mice, where the stimulation of B cell division is very evident on that day [2].

4. Discussion

Before discussing the nature of the cells responding to the various mitogens used, a comment should be made about the thymectomized, lethally x-irradiated, reconstituted animals. Analyses of the chimaeras bearing three karyotypic markers showed the existence of some host cell mitoses in all instances. In addition, when spleen cultures of chimaeras reconstituted with anti- Θ -treated bone marrow cells only, without thymocytes, were stimulated with PHA, various degrees of responsiveness were observed. Karyotypic analyses of these cultures showed that on the second day, more than half of the mitoses

were of host origin. On the third day, a majority of bone marrow-derived mitoses was observed, strongly suggesting that the PHA response resulted from the triggering of residual host T cells, followed by stimulation of the division of bone marrow-derived cells. This shows that mice, thymectomized at 4–8 weeks of age, lethally x-irradiated and bone marrow reconstituted, should not be considered as a model for the study of B lymphocyte function, since they may contain enough residual host T cells to generate, under strong stimulation, a T cell-dependent type of response.

Among the "nonspecific" mitogens explored, PWM was found not only to stimulate T and B lymphocytes independently of each other, *i.e.* in the absence of the other cell type, in agreement with results described by Janossy and Greaves [16], but also to stimulate them simultaneously and equally in cultures of chimaeras. In this situation, proliferation started with both cell types together and there was no marked predominance of one type of mitosis over the other at any time observed. RaMlg antiserum induced a moderate proliferation of B cells from the spleen, but no stimulation of CRT. It thus appears that only cells bearing surface immunoglobulins detectable by immunofluorescence [6, 17, 18] were stimulated by anti-Ig antisera. Finally, LPS, which has been reported to be a nonspecific B cell mitogen [19], indeed stimulated T-depleted spleen cells while having no effect at all on CRT. Moreover, mitoses induced in cultures of chimaeras were predominantly B. However, in view of the strong stimulation obtained, the question may be raised whether the highest percentages of T mitoses observed on the second and third day of culture, respectively 16 and 19 %, do not suggest some stimulation of T cells. This possibility receives strong support from the results of cultures from mice pretreated *in vivo* with vinblastine (discussed below), since a percentage of T mitoses as high as 50 % was observed on the 3rd day of a strongly stimulated culture. It seems likely, therefore, that the *in vitro* response of lymphoid cells to LPS is B cell-dependent, but that the stimulated cells [20], or perhaps the macrophages present in the spleen suspension [21], release factors able to stimulate some T cells. Whether these postulated factors require the presence of the mitogen to stimulate T cells and whether this stimulation can be transferred by the supernatant of LPS-stimulated culture, has not yet been explored.

Among the three types of antigens used to stimulate the cultures, only the bacteriophage T4 gave good stimulation with

Table 4. Effect of *in vivo* treatment with vinblastine^{a)} on cultures^{b)} of chimaeras

		Chimaeras ^{c)}	Stimulating agents								
			Back-ground	PHA		LPS		T4		SRBC	
				(cpm)	(cpm)	(% T)	(cpm)	(% T)	(cpm)	(% T)	(cpm)
A. Chimaeras T/H/BM not treated with vinblastine	1)	9800	85000	80	92000	16	N.D. ^{d)}		—	—	
	2)	7000	107000	70	71000	19	35800	15	—	—	
B. Chimaeras of the same group treated with vinblastine	3)	2700	100000	92	43600	50	N.D.		—	—	
	4)	5600	122000	92	43000	34	39300	59	—	—	
C. Chimaeras ^{e)} SRBC-primed treated with vinblastine	5)	4100	—	—	—	—	—	—	29000	92	
	6)	3400	—	—	—	—	—	—	10000	84	

a) Vinblastine (0.06 mg) was injected three times at 8 h intervals, with sacrifice 8 h later.

b) Cultures were arrested on day 3.

c) 1–6 indicate individual chimaeras, from which different types of cultures were established. Chimaeras 1 to 4 belonged to the same group.

d) N.D. = not done.

e) Chimaeras 5 and 6, primed with SRBC, had no "controls", that is no chimaeras of the same group not treated with vinblastine.

the spleen of unprimed animals under the culture conditions used. The mitotic response of unprimed mice to phage T4 was largely of B cell-type on the third day of culture, while parallel experiments indicated the T cell independence of the response, as well as the failure of T4 to stimulate CRT. It seems that some T cells can also become stimulated during the spleen cell culture, since the percentage of T mitoses was in some cases as high as 28 % and, with the spleen of a chimera treated *in vivo* with vinblastine, reached more than 50 %. In the cultures established from chimaeras previously immunized with T4, the mitotic response was stronger and consistently showed a higher percentage of T mitoses than cultures of unprimed mice. This very probably reflects the existence of primed T cells and would thus indicate that, *in vivo* too, the response to a clearly "thymus-independent" antigen such as phage T4 can involve T cell stimulation. Using a different experimental approach, Kruger and Gershorn reached a similar conclusion with some other "thymus-independent antigens" [22].

With the three antigenic systems explored, spleen cultures of immunized mice in the presence of immunizing antigens showed basic similarities in their responses.

1) The mitotic response was mainly of B cell type with, however, a clear participation of T cells, the highest percentages of T mitoses observed ranging from about 40 to 50 %, depending upon the antigen.

2) There was no indication that the T cells were stimulated to divide before the B cells. On the contrary, the majority of cultures arrested on sequential days showed more T mitoses on the third than on the second day of culture. This, however, does not necessarily mean in itself that B cells can start proliferating independently of any effect of the antigen on T cells, since there is evidence that the capacity of primed T cells to divide could be more limited than that of native T cells [23] and since the "helper effect" of T cells does not seem to require cell division [24, 25].

3) Destruction of T cells prior to culture by anti-MSLA + C showed the existence of a T cell-independent stimulation. The strongest T cell-independent response was observed in cultures performed in FCS with SRBC stroma, but in this instance, the role of a factor in FCS replacing the T cell function for SRBC could not be excluded [14]. Nevertheless, some degree of stimulation in the absence of T cells was also observed in rat serum and not only with SRBC stroma, but with xenogenic lymphoid cells as well.

The following conclusions can be drawn from these observations made on cultures of immunized animals. 1) It is erroneous to consider the *in vitro* stimulation by the immunizing antigen of cells from primed animals as reflecting uniquely, or even mainly, a T cell response. 2) Even with antigens considered to be "thymus-dependent", B cells can be stimulated to divide in the absence of T cells during an *in vitro* "secondary" response. This stimulation presumably would not lead to differentiation into antibody-producing cells, since, in the case of SRBC at least, the presence of T cells seems necessary for the appearance of antibody-forming cells [25], but it might result in an increase in antigen-binding cells. It has been observed *in vivo* that T cell-deprived mice, which are unable to make antibodies after stimulation with a "thymus-dependent" antigen, may nevertheless show, under these conditions, a definite increase in antigen-binding cells to this antigen [27].

The results obtained with xenogenic lymphoid cells stress the difference between the immune reaction to xenogenic and allo-

genic cells [28]. It was reported in a previous paper [2] that MLC of chimaeras immunized against the allogenic spleen cells used in the culture sometimes showed a percentage of T mitoses as low as 36 to 37 % on days 2 and 3; more often, however, there was an early wave of T mitoses reaching 90 % on these days, *i.e.* a response similar to that observed in MLC of non-immunized mice [2]. This last situation was not observed in six cultures with xenogenic cells, where 38 % of T mitoses was the highest, and not the lowest, proportion of T mitoses counted. Thus, the *in vitro* "secondary" response to xenogenic lymphoid cells resembles the secondary response observed with other antigens, while there is something unique in the response to allogenic lymphoid cells.

Injection of vinblastine *in vivo* for 36 h before the establishment of cell culture resulted in a markedly increased percentage of T mitoses on the third day of cultures stimulated by LPS or phage T4, or by SRBC stroma (primed mice). Such vinblastine treatment apparently results in the death of a rather important proportion of the cells dividing rapidly in the spleen, probably by arrest in metaphase. It would, therefore, appear that part of the B cells which are stimulated to divide *in vitro* belong to the progeny of cells dividing rapidly *in vivo*. A vinblastine-induced decrease in this last population would lead to a relative increase in the T cells dividing *in vitro*, and thus unmask or accentuate the participation of T cells in the *in vitro* proliferation. The population of B cells which is stimulated to divide by PHA and which becomes the predominant dividing population on the fourth day of culture [2], must also belong to the progeny of cells rapidly dividing *in vivo*, since in two chimaeras treated with vinblastine, there was persistence of a strong predominance of T mitoses on the 4th day of PHA-treated cultures.

Supported by grant No. 3.408.70 of the Fonds National Suisse de la Recherche Scientifique.

We thank Mrs. E. Bouchardy for her excellent technical assistance.

Received March 6, 1973.

5. References

- 1 Davies, A.J.S., *Transplant. Rev.* 1969. 1: 43.
- 2 Piguet, P.-F. and Vassalli, P., *J. Exp. Med.* 1972. 136: 962.
- 3 Schimpl, A. and Wecker, E., *Nature-New Biol.* 1972. 237: 13.
- 4 Britton, S., *Scand. J. Immunol.* 1972. 1: 89.
- 5 Andersson, J., Möller, G. and Sjöberg, O., *Eur. J. Immunol.* 1972. 2: 99.
- 6 Lamelin, J.P., Lisowska-Bernstein, B., Matter, A., Ryser, J.E. and Vassalli, P., *J. Exp. Med.* 1972. 136: 984.
- 7 Blomgren, H. and Andersson, B., *Cell. Immunol.* 1970. 1: 545.
- 8 Moorhead, P.S. and Nowell, P.C., in Eisen, H.N. (Ed.), *Methods in Medical Research*, Year book Medical Publishers, Chicago 1964, p. 310.
- 9 Dickson, R.C., Barnes, S.L. and Eislerling, F.A., *J. Mol. Biol.* 1970. 53: 461.
- 10 Doenhoff, M.J., Davies, A.J.S., Leuchars, E. and Wallis, V., *Proc. Roy. Soc. Lond. B.* 1970. 176: 69.
- 11 Adler, W.H., in Smith, R.T. and Landy, M. (Eds.), *Immunological surveillance*, Academic Press, New York-London 1970, p. 292.
- 12 Matter, A., Lisowska-Bernstein, B., Ryser, J.E., Lamelin, J.P. and Vassalli, P., *J. Exp. Med.* 1972. 136: 1008.
- 13 Adler, W.H., Takigushi, T., Marsh, B. and Smith, R.T., *J. Exp. Med.* 1970. 131: 1049.
- 14 Byrd, W., *Nature-New Biol.* 1971. 231: 280.

- 15 Valeriote, F.A., Bruce, W.R. and Meeker, B.E., *Biophys. J.* 1966. 6: 145.
- 16 Janosy, G. and Greaves, M.F., *Clin. Exp. Immunol.* 1972. 10: 525.
- 17 Raff, M.C., Sternberg, M. and Taylor, R.B., *Nature* 1970. 225: 553.
- 18 Pernis, B., Forni, L. and Amante, L., *J. Exp. Med.* 1970. 132: 1001.
- 19 Andersson, J., Sjöberg, O. and Möller, G., *Transplant. Rev.* 1972. 11: 131.
- 20 Vischer, T., *J. Immunol.* 1972. 109: 401.
- 21 Gery, I., Gershon, R.K. and Waksman, B.H., *J. Exp. Med.* 1972. 136: 143.
- 22 Kruger, J. and Gershon, R.K., *J. Immunol.* 1971. 106: 1065.
- 23 Sprent, J. and Miller, J.F.A.P., *Nature-New Biol.* 1971. 234: 195.
- 24 Kettman, J. and Dutton, R.W., *Proc. Nat. Acad. Sci. US.* 1971. 68: 699.
- 25 Hamaoka, T., Katz, D.H. and Benacerraf, B., *Proc. Nat. Acad. Sci. US* 1972. 69: 3453.
- 26 Chan, E.L., Mishell, R.I. and Mitchell, G.F., *Science* 1970. 170: 1215.
- 27 Roelants, G.E. and Askonas, B.A., *Nature-New Biol.* 1972. 239: 63.
- 28 Wilson, D.B. and Fox, D.H., *J. Exp. Med.* 1971. 134: 857.

